Cross down-regulation of leptin and insulin receptor expression and signalling in a human neuronal cell line

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Leptin and insulin are major signals to the hypothalamus to regulate energy homoeostasis and body adiposity. IR (insulin receptors) and leptin receptors (long isoform, ObRb) share a number of signalling cascades, such as JAK2/STAT-3 (Janus kinase 2/signal transduction and activator of transcription 3) and PI3K (phosphoinositide 3-kinase); the cross-talk between IR and ObRb have been described previously in non-neuronal cells. Differentiated human neuroblastoma (SH-SY5Y) cells express endogenous ObR and IR, and respond to leptin and insulin with stimulation of STAT-3 and MAPK (mitogen-activated protein kinase) phosphorylation, and PI3K activity. Insulin or leptin pre-treatment of SH-SY5Y cells increased basal STAT-3 phosphorylation, but abolished the acute effect of these hormones, and, interestingly, leptin pre-treatment abolished insulin effect and vice versa. Similar results were obtained for MAPK phosphorylation, but leptin or insulin pre-treatment did not completely abolish the acute effect of insulin or leptin. We have also showed that insulin and leptin are able to activate PI3K through IRS-1 (insulin receptor substrate 1) and IRS-2 respectively. Furthermore, leptin or insulin pre-treatment increased basal PI3K activity and IRS-1 or IRS-2 association with p85 and abolished acute insulin or leptin effect, in addition to the down-regulation of IRS-1 and IRS-2. Finally, insulin pre-treatment reduced leptin binding by approx. 60 %, and leptin pre-treatment reduced the expression of insulin receptor by 40 % in SH-SY5Y cells, which most likely accounts for the cross down-regulation of leptin and insulin receptors. These results provide evidence to suggest cross down-regulation of leptin and insulin receptors at both receptor and downstream signalling levels. This finding may contribute to the understanding of the complex relationship between leptin resistance and insulin resistance at the neuronal level.

Key words: insulin, insulin receptor substrate (IRS), leptin, leptin receptor (ObR), neuron, signal transduction and activator of transcription 3 (STAT-3).

INTRODUCTION

Leptin and insulin are major signals to the hypothalamus that regulate energy homoeostasis and body adiposity [1–3]. In hypothalamus, insulin and leptin act through their respective receptors, mainly located in the arcuate nucleus, and are able to reduce food intake and body weight in lean, but not in obese, subjects [4]. Whereas the effect of leptin in the hypothalamus involves the ObRb (long isoform of the leptin receptor), the role of insulin has been suggested by the observation that insulin-deficient animals are hyperphagic and the administration of insulin in the third ventricle normalizes their food intake and body weight [5–7]. In the hypothalamus, insulin and leptin act on POMC (pro-opiomelanocortin) neurons by activating the secretion of an anorexic neuropeptide, α-melanocyte-stimulating hormone [8,9], and on NPY/AGRP (neuropeptide Y/agouti gene-related protein) neurons by inhibiting the expression of the orexic neuropeptide NPY [10]. It is noteworthy that both central leptin and insulin resistance can lead to hyperphagia, increased plasma insulin and leptin concentrations, and changes in energy balance and fat mass [11–13].

The IR (insulin receptor) belongs to the tyrosine kinase receptor family, and its autophosphorylation creates a binding site for a family of at least four closely related intracellular signalling molecules known as IRS-1–IRS-4 (IR substrates 1–4) [14]. IRS-2 and IRS-4 are particularly expressed in the hypothalamic arcuate nucleus [13]. Phosphorylated IRS proteins bind and activate further downstream signalling proteins, such as PI3K (phosphoinositide 3-kinase) and PKB (protein kinase B) [14]. In addition, IR is able to phosphorylate other substrates, such as Shc (Src-homology collagen), through its PTB domain (phosphotyrosine-binding domain) [15].

The leptin receptor belongs to the cytokine receptor superfamily [16]. Several different leptin receptor isoforms exist, including a long form (ObRb), which is highly expressed in the hypothalamus, and a short form (ObRa), which is highly expressed in microvessels of the blood–brain barrier [16–18]. Upon leptin binding ObRb activates JAK2 (Janus kinase 2), which in turn phosphorylates tyrosine residues in the receptor tails, leading to recruitment and activation of signalling molecules [19,20]. Among these, the STAT-3 (signal transducer and activator of transcription 3) directly transmit the signals to the nucleus [21–23]. Phosphorylated STAT-3 proteins dimerize and translocate to the nucleus where they bind to specific nucleotide sequences and induce gene expression. The negative control of this signalling pathway is provided by a family of SOCS (suppressor of cytokine signalling) molecules that are induced in response to STAT-3 phosphorylation [24,25]. The leptin receptor, through the activation of JAK2, is also able to phosphorylate IRS proteins and induce the IRS–PI3K signalling pathway [26–29]. The overlapping effects of insulin and leptin have been extended to hypothalamus, where insulin modulates leptin-induced STAT-3 tyrosine phosphorylation [30]. Several lines of evidence suggest that the central nervous system may be a critical target for leptin action, by which it maintains euglycaemia without affecting insulin secretion [31,32]. In fact, several *in vivo*

Abbreviations used: α, antibody against; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IR, insulin receptor(s); IRS-1, insulin receptor substrate; JAK2, Janus kinase 2; (h)LBD, (human) ligand-binding domain; MAPK, mitogen-activated protein kinase; NeuroD-1, neurogenic differentiation 1; ObR(b), leptin receptor (long form); PI3K, phosphoinositide 3-kinase; RA, retinoic acid; RT-PCR, reverse transcriptase PCR; SHP, SH2-domain-containing tyrosine phosphatase; STAT-3, signal transduction and activator of transcription 3.

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studies have shown that leptin treatment not only enhances glucose turnover in normal rodents [33,34], but also ameliorates impaired glucose metabolism in leptin-deficient *ob*/*ob* mice [35]. These data suggest that leptin and insulin in the hypothalamus and probably in neuronal cells may have overlapping effects in the control of food intake. However, the direct cross talk between IR and ObRb signalling pathways has not yet been clearly demonstrated in neuronal cells. In addition, conflicting results have been reported in studies concerning the interaction of leptin and insulin signal transduction in a hepatoma cell line [10,29,36]. Thus in the present paper we aimed to dissect the possible cross-talk between ObRb and IR at the level of three major signalling pathways, STAT-3, MAPK (mitogen-activated protein kinase) and PI3K in differentiated SH-SY5Y human neuroblastoma cells [37] which express endogenous IR and ObRb. Both leptin and insulin activate STAT-3 and MAPK phosphorylations, but the chronic treatment with leptin or insulin showed that these pathways behaved differently. The STAT-3 pathway was clearly desensitized, but MAPK pathway was not. Leptin treatment resulted in the recruitment of p85 to ObRb, suggesting the formation of a multimeric complex, most likely involving ObRb–JAK-2–IRS-2–p85. Finally, we showed that a chronic treatment with leptin or insulin led to a clear down-regulation of IR and ObRb respectively, which may be considered as a major finding that could contribute to understanding of the link between leptin resistance and insulin resistance in neuronal cells.

EXPERIMENTAL

Chemicals and materials

BSA (fraction V radio immunoassay grade), leupeptin, aprotinin and Protein A–agarose were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Pre-made polyacrylamide solution Protogel was from National Diagnostics (Prolabo, Paris, France). Antibodies directed towards IRS-1, IRS-2, total STAT-3, phospho-STAT-3, IR, anti-phosphotyrosine (clone 4G10) were purchased from UBI (Euromedex, Souffelweyersheim, France); antibodies against p44/p42 MAPK and phospho-p44/p42 MAPK were from Cell Signaling (Ozyme, France) and antibodies against p85 were from Santa Cruz Biotechnology (Tebu, France). [γ^{32}-P]ATP was obtained from Amersham. Nitrocellulose membranes were from Euromedex.

Cell culture

SH-SY5Y human neuroblastoma cells (kindly provided by Dr B. Dufy, CNRS UMR 5543, Universit Victor Segalen Bordeaux 2, Bordeaux, France) were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % heat-inactivated foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in 5 % CO_2 atmosphere at 37 °C; differentiation of SH-SY5Y cells was achieved by treatment with RA (retinoic acid) [37]. Differentiated cells were used after 15 days of RA treatment to obtain a high percentage of cells that showed a clear morphological differentiation.

Iodination of sheep leptin

Sheep leptin (10 μg) was radio-iodinated (IMS 50, Amersham Pharmacia Biotech) by the chloramine T method, as described previously [38].

^{125}I-Leptin-binding assay

SH-SY5Y cells were serum deprived for 16 h, and incubated with 500 000 c.p.m. of ^{125}I-leptin in PBS containing 0.1 % BSA at room temperature for 4 h, in the presence or the absence of unlabelled leptin (1 μg/ml). Cells were then washed three times with binding medium (PBS/BSA, 0.1 %), and the radioactivity associated with the cells was measured with a γ-radiation counter.

Immunocytochemistry

SH-SY5Y cells were grown in DMEM supplemented with 10 % foetal bovine serum. After washing in PBS, the cells were fixed for 30 min in 2 % paraformaldehyde in PBS, and washed three times in PBS. After blocking in 5 % normal goat serum and 0.2 % gelatine fish, samples were incubated with a rabbit polyclonal antibody purified on Protein A column (Perbio Science, Bezons, France) developed in our laboratory (Ab220), raised against a portion of the LBD (ligand-binding domain) of human leptin receptor [39], at a 1:50 dilution overnight at 4 °C. The negative immune control for this antibody was an equivalent concentration of non-immune rabbit Ig substituted for the primary antibody at an equivalent dilution. After washing, the cells were incubated with FITC goat anti-rabbit IgG (1:400; Vector Laboratories, Burlingame, CA, U.S.A.) and washed again. All samples were briefly counterstained with DAPI (4,6-diamidino-2-phenylindole) at 1:300 dilution to count the number of nuclei per field of view. Cells were mounted and cover-slips and anti-fade medium were added (Vector Laboratories). Cell analysis was carried out using a DMRB microscope (Leica Microsystems) equipped with a mercury light source and filter system to visualize the green immunofluorescence.

Determination of ObRb, NeuroD-1 (neurogenic differentiation 1), p85 and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA expression using RT-PCR (reverse transcriptase PCR)

Following RNA extraction from SH-SY5Y cells, total RNAs were subjected to RT-PCR as described previously [40]. The PCR primers were as follows: NeuroD-1, 5′ primer, 5′-CTCAGTTCTCAGGACGAGGA-3′; 3′ primer, 5′-GATCTCTGACAGAGCCCAGA-3′ (flanking a region of 390 bp); ObRb, 5′ primer, 5′-ACACTGTTAATTTCACACCAGAG-3′; 3′ primer, 5′-TGGATAAACCCTTGCTCTTCA-3′ (flanking a region of 445 bp); p85, 5′ primer, 5′-CGCGGATCCCTTGCACTTGGGTGACATAT-3′; 3′-primer, 5′-CTAGTCTAGACAATGGCTTCCACGAGCTTG-3′ (flanking a region of 351 bp); GAPDH, 5′-primer, 5′-AAACCCATCACCATCTTCCAG-3′; 3′-primer, 5′-TCCCCGGTAGGTGTCAGAAGA-3′ (flanking a region of 360 bp). RT-PCR products were analysed by an agarose gel (1 %) electrophoresis, stained with ethidium bromide (1 mg/ml).

Cell stimulation, immunoprecipitation and immunoblotting

SH-SY5Y cells were starved in serum-free DMEM for 16 h, and stimulated with insulin (100 nM), leptin (15 nM) or with the combination of both hormones. At the indicated times after stimulation, cells were harvested by rinsing in ice-cold PBS and scraping into lysis buffer containing 20 mM Tris/HCl (pH 7.5), 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 % Nonidet-P40, 10 % glycerol, protease inhibitors (0.35 mg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin) and phosphatase inhibitors (10 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM sodium β-glycerophosphate, 10 mM benzamidine). After lysis in ice for 30 min, insoluble materials was removed by centrifugation (17 000 ***g*** at 4 °C for 30 min), and protein concentrations of the resulting lysates were determined using a protein assay kit (Pierce). For immunoprecipitation, equal amounts of proteins were incubated with the appropriate antibodies overnight at 4 °C. Immune complexes were collected on to a mixture of Protein A–agarose and Protein G–agarose for 2 h at 4 °C, washed extensively, resolved by

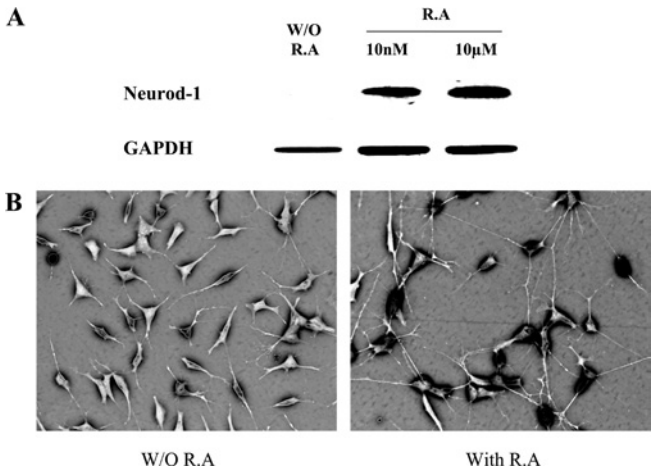

Figure 1 RA induces SH-SY5Y cell differentiation

Cells were cultured without (W/O) or with 10 nM or 10 μM retinoic acid for 15 days. (**A**) The expression NeuroD-1 was measured by RT-PCR in parallel with the expression of GAPDH. (**B**) Phase-contrast images of the undifferentiated cells (left panel) and differentiated cells following RA treatment (10 nM for 15 days; right panel).

SDS/PAGE and the proteins were transferred on to nitrocellulose membranes. Immunoblots were blocked with 3 % BSA or non-fat dried milk. After incubation with appropriate primary and secondary antibodies, nitrocellulose membranes were washed and targeted proteins were detected using enhanced chemiluminescence reagents (ECL®; Amersham Biosciences).

PI3K assay

Cell lysates (1 mg of protein) were subjected to immunoprecipitation with appropriate antibodies overnight at 4 °C, and PI3K was measured as described previously [41].

Statistical analysis

Statistical analysis was performed using ANOVA (Statview software program, version 5) to detect significant inter-group differences. The results are expressed as the means $\pm$ S.E.M., and $P < 0.05$ was considered statistically significant.

RESULTS

SH-SY5Y neuroblastoma cells express leptin receptors in an RA-dependent manner

RA, as reported previously [37], induces neural differentiation of SH-SY5Y neuroblastoma cells. We have confirmed RA-dependent differentiation of these cells by measuring differentiation-promoting NeuroD-1 gene expression. RA (10 nM or 10 μM) induces the expression of NeuroD-1, as estimated by RT-PCR and normalized to GAPDH expression (Figure 1A). The differentiation of SH-SY5Y cells was mirrored by a clear augmentation of neurite-bearing cells, as visualized by phase-contrast images (Figure 1B). SH-SY5Y cell differentiation has been described to be PI3K dependent [37]. This enzyme is highly regulated by the IR and recent reports [26–29] have also indicated its regulation by ObRb. Thus leptin receptor and IR expression was studied as compared with the expression of the catalytic subunit of PI3K (p85) during the SH-SY5Y differentiation process. RA induced the expression of ObRb at both 10 nM and 10 μM concentrations

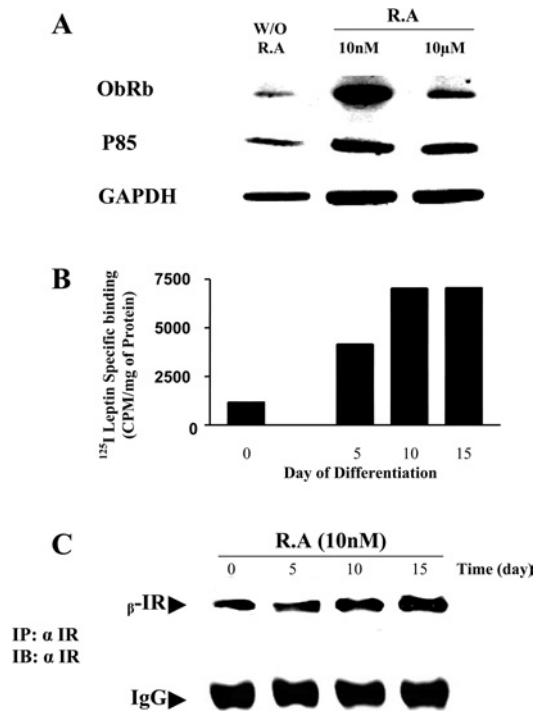

Figure 2 Expression of ObRb is increased during RA-induced differentiation

(**A**) The expression of ObRb and p85 subunit of PI3K were measured by RT-PCR using specific primers as described in the Experimental section, and the expression of GAPDH was used as positive control. The RT-PCR was performed on undifferentiated cells (W/O RA) and differentiated cells following addition of 10 nM or 10 μM RA. (**B**) The amount of cell surface leptin receptor was measured during differentiation (0, 5, 10 and 15 days) using ^{125}I-leptin, and the results are expressed as specific binding c.p.m./mg of total cell proteins. (**C**) Cell lysates (500 μg) from undifferentiated cells or cells cultured with 10 μM RA for 5, 10 or 15 days were immunoprecipitated with an anti-IR antibody and subjected to Western blotting, and the protein was revealed with the same antibody. The IgG band in the blot is shown as internal control. IB, immunoblot; IP, immunoprecipitation.

in parallel with the expression of p85, whereas the GAPDH expression was not affected (Figure 2A). The expression of ObRb was confirmed by an ^{125}I-leptin-binding study: leptin-specific binding was increased under RA treatment to reach its maximum after 10 days of treatment (at least 4-fold as compared with undifferentiated cells, Figure 2B). In addition, these findings were confirmed by the localization of leptin receptor on cultured undifferentiated and differentiated SH-SY5Y cells using immunocytochemistry. Specific antibodies (αhLBD) directed towards hLBD (human LBD) were used as described in the Experimental section. Cells were incubated in the presence of pre-immune serum or αhLBD. Leptin receptor labelling was present on both undifferentiated and differentiated cells, but with different intensities (Figure 3). The labelling clearly increased in differentiated (Figure 3A) as compared with undifferentiated (Figure 3B) cells. Leptin receptor immunoreactivity was associated with the plasma membrane (Figure 3). Leptin receptor labelling was also observed within the cytoplasm (Figure 3), which is likely to relate to intracellular sites of leptin receptor synthesis and metabolism.

In contrast, IR expression was not affected during differentiation, as shown by Western blotting using specific IR antibodies (Figure 2C).

As the present paper aims to study the cross-talk between leptin and insulin receptor signalling pathways, we choose to use differentiated SH-SY5Y cells that express both receptors. Therefore, all the following experiments were performed on differentiated cells.

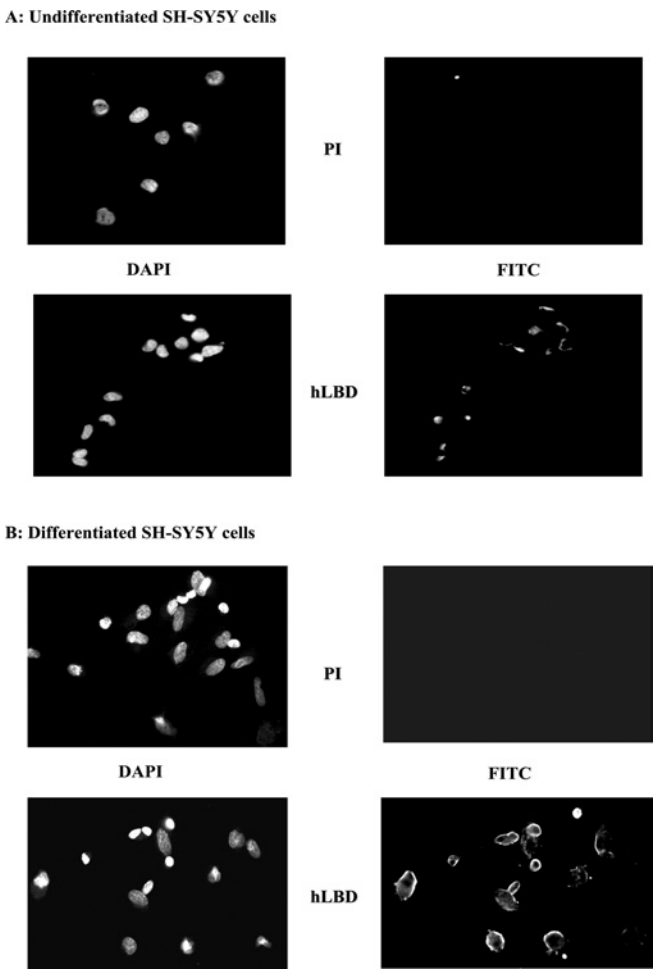

Figure 3 Localization of leptin receptor in undifferentiated and differentiated SH-SY5Y cells using immunochemistry

Undifferentiated (**A**) or differentiated (**B**) (following 15 days of RA treatment) cells were starved (16 h) then incubated in the presence of specific antibodies directed towards hLBD or of pre-immune serum (PI) used as negative control, as described in the Experimental section. DAPI, 4,6-diamidino-2-phenylindole.

SH-SY5Y cells express functional leptin and insulin receptors

SH-SY5Y cells were treated in the absence or presence of leptin or insulin or both hormones for 3 or 10 min. Cells were previously serum starved for 16 h. Following immunoprecipitation using specific αLBD or a pre-immune serum and Western blot, bands were revealed by anti-phosphotyrosine antibody. Leptin clearly induced the phosphorylation of two major bands of approx. 200 kDa and 120 kDa (Figure 4A, upper panel). The combination of both leptin and insulin did not further increase the phosphorylation of the two bands. However, insulin alone was without effect and no bands were revealed when the immunoprecipitation was performed with the pre-immune serum (Figure 4A, upper panel). The 200 kDa band most likely corresponds to ObRb. The 120 kDa band corresponds to JAK2 and this was confirmed following re-blotting using an anti-JAK2 antibody (Figure 4A, lower panel). Thus co-immunoprecipitated JAK2 and ObRb are phosphorylated in a leptin-dependent manner, and insulin does not affect this phosphorylation. To investigate the functionality of IR, cells were treated as described above and, following immunoprecipitation with anti-IR antibodies (αIR) and blotting with anti-phosphotyrosine antibodies, the phosphorylated β subunit of the IR was revealed. Insulin clearly induced the phosphorylation of IR, but

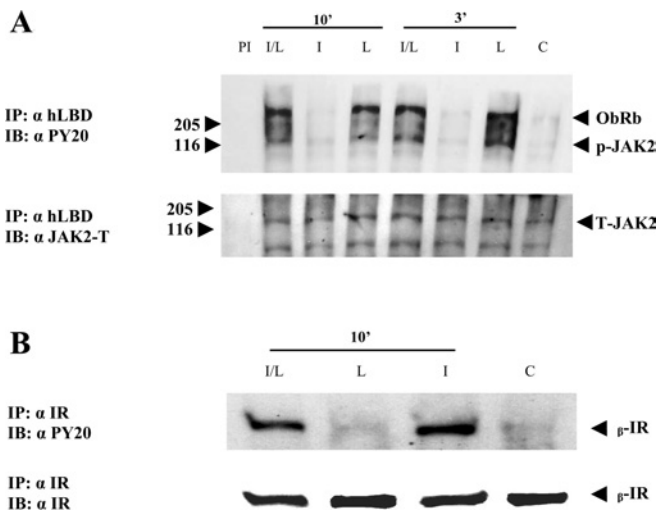

Figure 4 ObRB and IR phosphorylation in differentiated SH-SY5Y cells

Cells were serum deprived for 16 h then incubated for 3 or 10 min in the presence or absence of leptin (L), insulin (I) or insulin plus leptin (I/L). Following solubilization, cell lysates were: (**A**) immunoprecipitated with an anti-ObR antibodies and blots were revealed with anti-phosphotyrosine antibodies (upper panel) or anti-JAK-2 antibodies (lower panel); (**B**) immunoprecipitated with anti-insulin receptor antibodies and blotted with anti-phosphotyrosine antibodies (upper panel) or anti-IR antibodies (lower panel). All blots were revealed by ECL®. IB, immunoblot; IP, immunoprecipitation.

not leptin, and the combination of both insulin and leptin did not further increase IR phosphorylation (Figure 4B, upper panel). The re-blotting with αIR indicated the total amount of IR expressed in cells (Figure 4B, lower panel).

Cross-desensitization of ObRb- or IR-dependent STAT-3 tyrosine phosphorylation following insulin or leptin pre-treatment in SH-SY5Y cells

To investigate the possible cross-regulation of JAK2–STAT-3 signalling pathway by leptin and insulin receptors, SH-SY5Y cells were pre-treated with or without leptin (15 nM) or insulin (100 nM) for 16 h and then incubated for 10 min in the absence or presence of leptin, insulin or leptin plus insulin, as described in the Experimental section. Western blot analysis of cell lysates was performed using an anti-phospho-STAT-3 or an anti-STAT-3 antibody, and the results were expressed as the ratio of phosphorylated STAT-3 and total STAT-3. In untreated cells (starved cells), both leptin and insulin significantly increased STAT-3 phosphorylation, and the combination of leptin and insulin further increased STAT-3 phosphorylation as compared with insulin alone (Figure 5A). In cells pre-treated with insulin, basal STAT-3 phosphorylation was increased and acute incubation with leptin or insulin did not further increase STAT-3 phosphorylation (Figure 5B). A similar effect was observed in cells pre-treated with leptin, but with some differences. Leptin pre-treatment increased the basal level of STAT-3 phosphorylation, but to a lesser extent, as compared with insulin pre-treatment. In addition, the combination of insulin and leptin in the acute stimulation significantly increased STAT-3 phosphorylation, which is not the case in cells pre-treated with insulin (Figure 5C).

Partial cross-desensitization of ObRb- or IR-dependent MAPK phosphorylation following insulin or leptin pre-treatment in SH-SY5Y cells

The MAPK pathway could also be shared by insulin and leptin receptor signalling cascade, and may therefore be subjected to a

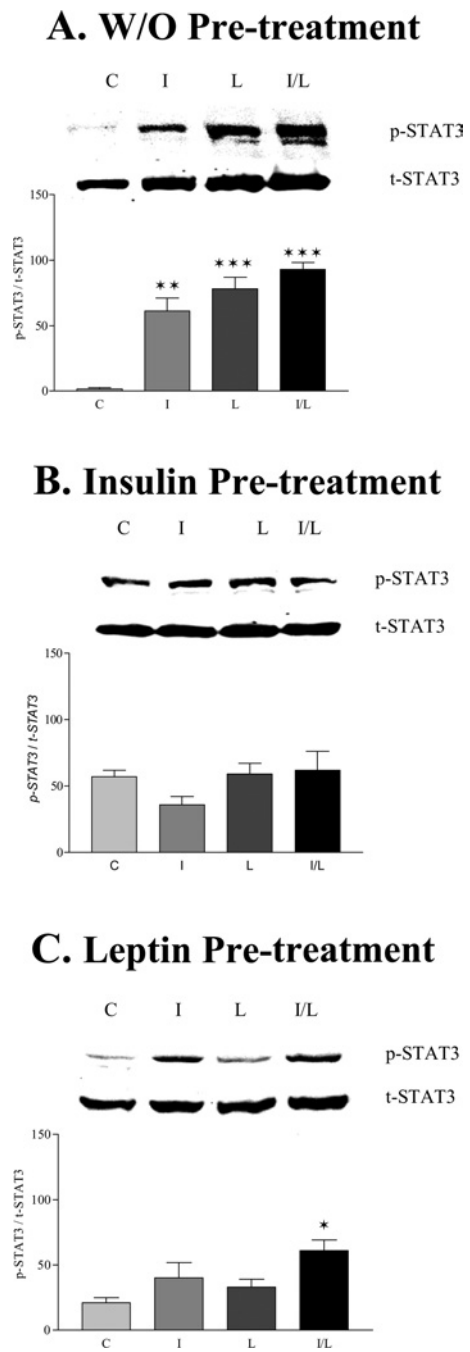

Figure 5 Chronic effects of insulin and leptin on STAT-3 tyrosine phosphorylation in SH-SY5Y cells

Serum-deprived cells were incubated in the absence (C) or presence of insulin (I), leptin (L) or insulin plus leptin (I/L), following a 16 h pre-treatment without (**A**) or with insulin (**B**) or leptin (**C**). Following solubilization, cells lysates were subjected to Western blotting, and blots were incubated with anti-phospho-STAT-3 antibodies (p-STAT-3) and, following stripping, with anti-STAT-3 antibodies (t-STAT-3). The proteins on the blots were revealed by ECL®, the results were expressed as the p-STAT-3/t-STAT-3 ratio and presented as means ± S.E.M. ($n = 4$). *, ** and *** indicate $P < 0.01$, $P < 0.001$ and $P < 0.0001$ respectively.

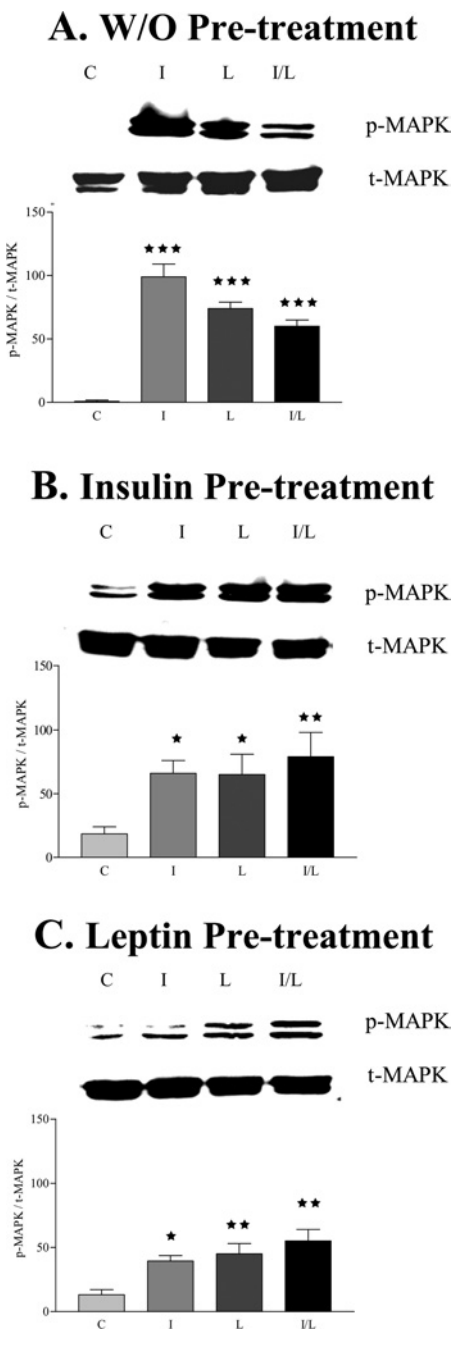

Figure 6 Chronic effects of insulin and leptin on MAPK phosphorylation in SH-SY5Y cells

Serum-deprived cells were incubated in the absence (C) or presence of insulin (I), leptin (L) or insulin plus leptin (I/L), following a 16 h pre-treatment without (**A**) or with insulin (**B**) or leptin (**C**). Following solubilization, cells lysates were subjected to Western blotting, and the blots were incubated with anti-phospho-MAPK antibodies (p-MAPK) and, following stripping, with anti-MAPK antibodies (t-MAPK). The proteins on the blots were revealed by ECL® and the results were expressed as the p-MAPK/t-MAPK ratio and presented as the means ± S.E.M. ($n = 4$). *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.0001$ respectively.

cross-regulation involving leptin and insulin. Using an identical protocol as described above, cells were pre-treated or not with leptin or insulin and then MAPK phosphorylation was measured in response to acute stimulation with these hormones. In non-pre-treated cells, both leptin and insulin induced MAPK phosphorylation, and the combination of both leptin and insulin did not further increase MAPK phosphorylation (Figure 6A), and a tendency towards reduction was observed. Both insulin (Figure 6B) and leptin (Figure 6C) pre-treatments increased the basal phosphorylation of MAPK, but reduced the amplitude of response to acute stimulation by insulin or leptin. However, insulin pre-treatment did not abolish the acute effects of insulin or leptin on MAPK

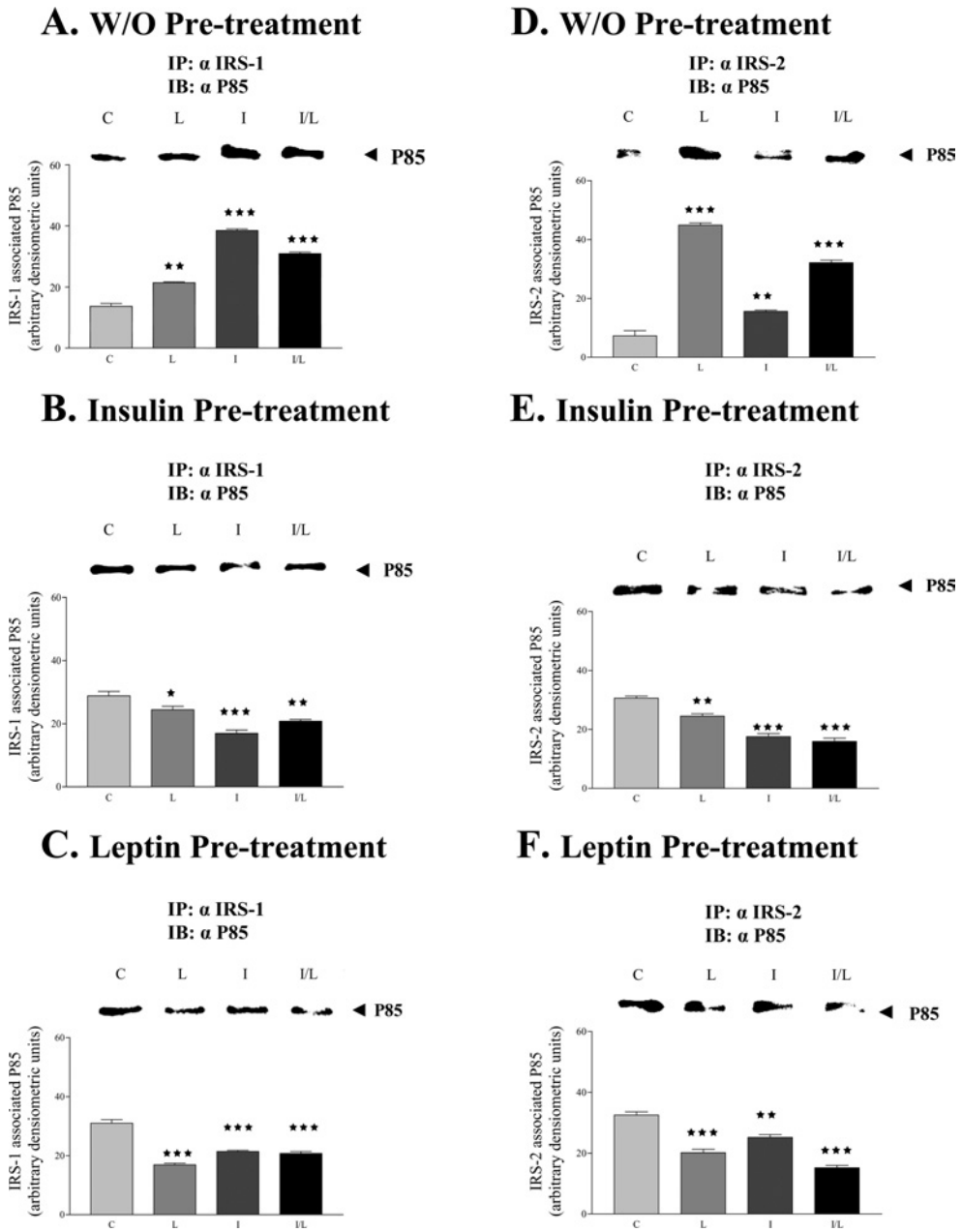

Figure 7 Effects of chronic leptin or insulin treatment on IRS-1 or IRS-2 association with p85 subunit of PI3K

Serum-deprived cells were incubated in the absence (C) or presence of insulin (I), leptin (L) or insulin plus leptin (I/L) for 10 min following a 16 h pre-treatment without (**A**, **D**) or with insulin (**B**, **E**) or leptin (**C**, **F**). Following solubilization, cells lysates were immunoprecipitated with anti-IRS-1 antibodies (**A**–**C**) or with anti-IRS-2 antibodies (**D**–**F**) and subjected to Western blotting. The blots were incubated with anti-p85 antibodies and revealed by ECL® and bands corresponding to p85 were quantified by Scion Image software. The results are expressed as the means ± S.E.M. ($n = 4$). *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.0001$ respectively. IB, immunoblot; IP, immunoprecipitation.

phosphorylation (Figure 6B), whereas leptin pre-treatment markedly reduced the efficacy of leptin or insulin in phosphorylating MAPK, but their effect still remained significant as compared with unstimulated cells (Figure 6C).

Insulin and leptin activate IRS (1 or 2) association to p85 subunit and PI3K activity in SH-SY5Y cells

Recent evidence has attributed a key role to the PI3K in mediating the anorexic effect of leptin and insulin in the hypothalamus. These studies have shown that leptin, as for insulin, activates PI3K signalling, providing a plausible mechanism for neuronal cross-talk between insulin and leptin signalling. We firstly investigated the activation of PI3K in response to insulin and leptin in SH-SY5Y cells. Following stimulation of SH-SY5Y cells by insulin, leptin or both for 10 min, cells were solubilized and lysates immunoprecipitated with either IRS-1 or IRS-2 antibodies and blots were revealed with anti-p85 antibodies (regulatory subunit of PI3K). Insulin and leptin significantly increased the association of IRS-1 to PI3K, with a insulin producing a larger effect, and the combination of leptin and insulin did not further increase this association (Figure 7A). The association of IRS-2 to p85 was also significantly increased by leptin and insulin. However, the level of leptin-induced IRS-2–p85 association was significantly higher as

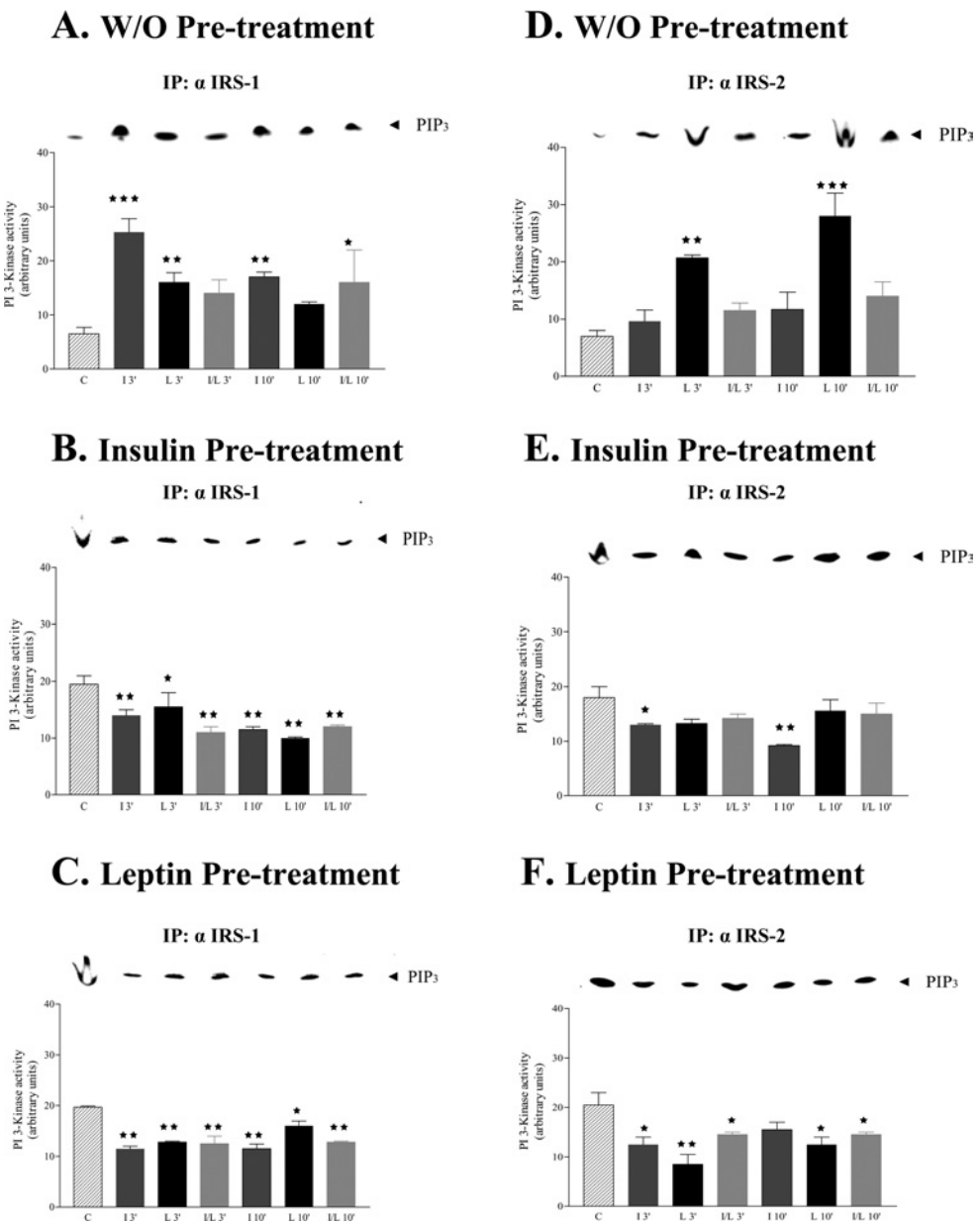

Figure 8 Effects of chronic leptin or insulin treatment on PI3K activity associated with IRS-1 or IRS-2

Serum-deprived cells were incubated for 3 or 10 min in the absence (C) or presence of insulin (I), leptin (L) or insulin plus leptin (I/L), following a 16 h pre-treatment without (**A**, **D**) or with insulin (**B**, **E**) or leptin (**C**, **F**). Following solubilization, cells lysates were immunoprecipitated with anti-IRS-1 antibodies (**A**–**C**) or with anti-IRS-2 antibodies (**D**–**F**). Immunoprecipitates were incubated with an artificial substrate for PI3K, phosphatidylinositol (PI), in the presence of [γ-^{32}P]dATP. The inserts present a representative experiment indicating the phosphorylated PI (PIP_3). Phosphorylation of PI was quantified by STORM phosphoImager. The results are expressed as the means $\pm$ S.E.M. ($n = 4$). *, ** and *** indicate $P < 0.05$, $P < 0.01$ and $P < 0.0001$ respectively. IP, immunoprecipitation.

compared with the insulin effect (Figure 7D). These associations were confirmed by measuring PI3K activity associated to IRS-1 or IRS-2. At 3 or 10 min of stimulation, insulin seemed to preferably activate PI3K through IRS-1 (Figure 8A), whereas leptin clearly activates PI3K through IRS-2, but also via IRS-1 to a lesser extent (Figure 8D).

Cross-desensitization of ObRb- or IR-dependent IRS (1 or 2) association with p85 and PI3K activity following insulin or leptin pre-treatment in SH-SY5Y cells

We next, as described for JAK2/STAT-3 and MAPK pathways, investigated the effect of leptin or insulin pre-treatment on PI3K pathway. Insulin (100 nM) (Figure 7B) and leptin (15 nM) (Figure 7C) pre-treatments clearly abolished co-immunoprecipitation of IRS-1 and p85 in response to acute stimulation with insulin or leptin, as compared with non-pre-treated cells (Figure 7A). Furthermore, the association of IRS-1 with p85 was increased following both leptin or insulin pre-treatment and reduced following acute stimulation with insulin or leptin (Figures 7B and 7C). A similar pattern was observed for IRS-2 association to p85, where insulin (Figure 7E) and leptin (Figure 7F) pre-treatments abolished leptin-induced association of IRS-2 with p85 as compared with non-pre-treated cells (Figure 7D). As for the IRS-1 pathway, a negative effect of leptin on IRS-2–PI3K association was observed. In addition, the basal IRS-2–p85 association

was increased in response to leptin or insulin pre-treatment as compared with non-pre-treated cells (Figures 7E and 7F). The effect of leptin or insulin pre-treatment was confirmed by measuring PI3K activity associated with IRS-1 or IRS-2. The activation of PI3K associated with IRS-1 in response to insulin or leptin was completely abolished in cells pre-treated either with insulin (Figure 8B) or leptin (Figure 8C), as compared with non-pre-treated cells (Figure 8A). Both pre-treatments increased basal activity and, in addition, in leptin pre-treated cells insulin or leptin have a negative effect on PI3K activity (Figure 8C). Similar results were obtained when IRS-2-associated PI3K activity was measured. Both insulin (Figure 8E) or leptin (Figure 8F) abolished the effect of leptin on activating PI3K associated with IRS-2, and, in addition, insulin or leptin pre-treatment increased basal PI3K activity compared with non-pre-treated cells (Figure 8D).

Down-regulation of IRS-1 and IRS-2 following leptin or insulin pre-treatment in SH-SY5Y cells

Since leptin or insulin pre-treatment affected IRS-1 or IRS-2 association to p85 subunit and reduced PI3K activity in response to acute stimulation by leptin or insulin, we investigated the impact of such pre-treatment on IRS-1 and IRS-2 expression. Following insulin (100 nM) or leptin (15 nM) pre-treatment, IRS-1 (Figure 9, upper panel) and IRS-2 (Figure 9, lower panel) expression levels were measured after immunoprecipitation with αIRS-1 or αIRS-2 and immunobloting with the same antibody. Leptin or insulin pre-treatment significantly reduces IRS-1 (Figure 9, upper panel) and IRS-2 (Figure 9, lower panel) expression levels. In addition, we show that in non-pre-treated cells insulin preferentially phosphorylates IRS-1 (Figure 9, upper panel) and leptin preferentially phosphorylates IRS-2 (Figure 9, lower panel). Following pre-treatment, IRS-1 and IRS-2 phosphorylation in response to leptin or insulin was abolished, which is confirmed by the reduced association of IRS-1 (Figure 9, upper panel) or IRS-2 (Figure 9, lower panel) with the p85 subunit.

ObRb co-immunoprecipitates with p85 regulatory subunit of PI3K in SH-SY5Y cells

Leptin and insulin communication at the level of PI3K indicates that insulin receptor recruits PI3K most likely through IRS-1 and leptin receptor preferentially through IRS-2. The IR–IRS-1–PI3K pathway has been largely documented, whereas leptin receptor–IRS-2–PI3K association is not yet well established. Therefore to confirm the involvement of PI3K in leptin receptor signalling pathway, probably through IRS-2, the co-immunoprecipitation of p85 with ObRb was analysed, and in parallel we investigated co-immunoprecipitation of ObRb with IRS-2 or IRS-1. Leptin clearly increased the co-immunoprecipitation of ObRb with p85, whereas insulin had only a slight effect (Figure 10). In addition, leptin increases ObRb association with IRS-2 and to a lesser extent with IRS-1. Both leptin or insulin pre-treatment abolished these interactions (Figure 10).

Cross-down-regulation of ObRb or IR following insulin or leptin pre-treatment in SH-SY5Y cells

The effect of insulin or leptin pre-treatment altered the JAK2/STAT-3, MAPK and PI3K signalling pathways, and this could result from the alteration of earlier signalling steps, such as receptor expression or phosphorylation. Therefore, following leptin (15 nM) or insulin (100 nM) pre-treatment, leptin-specific binding was measured on intact cells using ^{125}I-leptin. Leptin or insulin pre-treatment reduced specific ^{125}I-leptin binding by about 85 % and 60 % respectively (Figure 11). The impact on IR density or

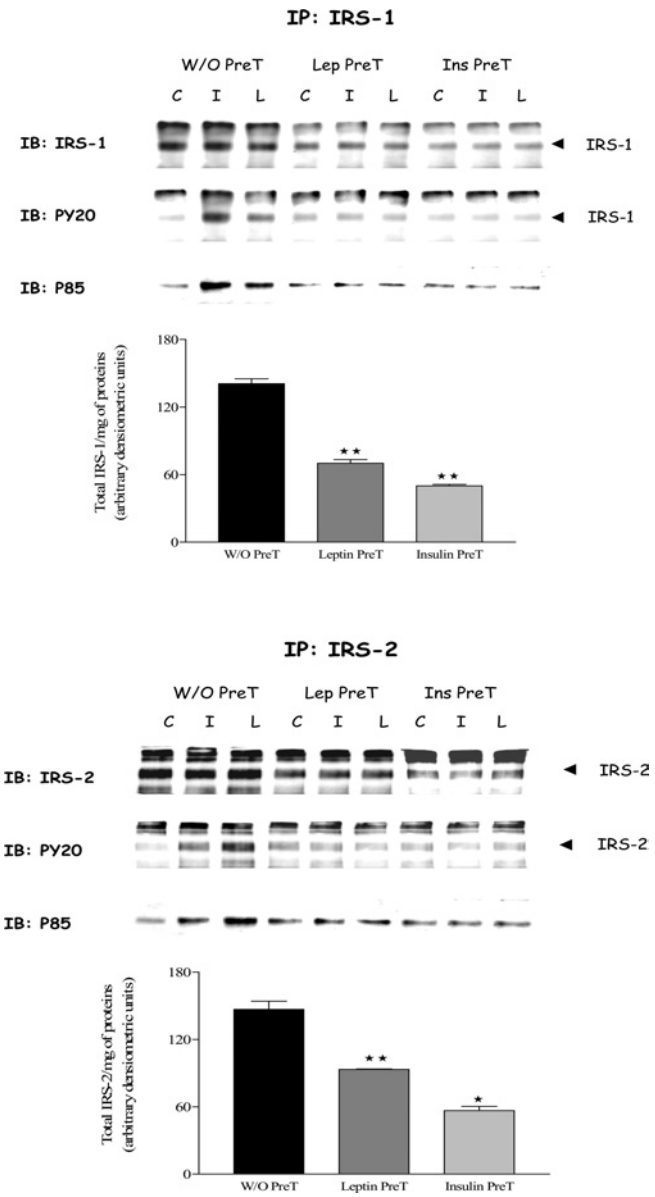

Figure 9 Effects of chronic leptin or insulin treatment on IRS-1 or IRS-2 expression and phosphorylation

Serum-deprived cells were incubated in the absence (C) or presence of insulin (I) or leptin (L) for 10 min, following a 16 h pre-treatment with or without insulin (I) or leptin (L). Following solubilization, cells lysates were immunoprecipitated with anti-IRS-1 antibodies (upper panel) or with anti-IRS-2 antibodies (lower panel), and subjected to Western blotting. The blots were incubated with antibodies directed towards: IRS-1, IRS-2, phosphotyrosine (PY20) or p85. The proteins were revealed by ECL® and bands corresponding to total IRS-1 or IRS-2 were quantified by Scion Image software. The results are expressed as the means $\pm$ S.E.M. ($n = 4$). * and ** indicate $P < 0.001$ and $P < 0.0001$ respectively. IB, immunoblot; IP, immunoprecipitation.

phosphorylation was also analysed. Insulin pre-treatment completely abolished IR phosphorylation in response to insulin (Figure 12) as compared with control cells, whereas leptin pre-treatment inhibited IR phosphorylation in response to insulin, but not totally (Figure 12). Leptin or insulin pre-treatment also reduced the co-immunoprecipitation of IR with p85 (Figure 12). The impact on IR expression was also examined by Western blot analysis using specific anti-IR antibody following immunoprecipitation with the same antibody. Insulin pre-treatment significantly reduced IR expression (approx. 80 %), and a similar effect, but to

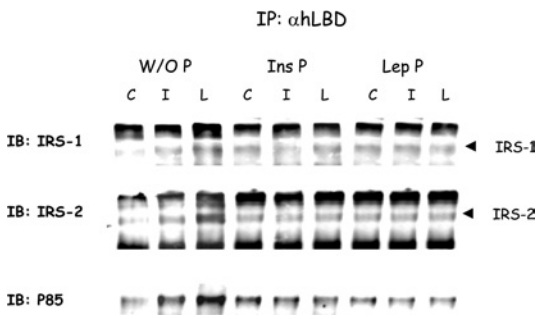

Figure 10 Co-immunoprecipitation of ObR with IRS-1, IRS-2 and p85 subunit of PI3K

Serum-deprived cells were incubated in the absence (C) or presence of insulin (I) or leptin (L) for 10 min following a 16 h pre-treatment without (W/O P) or with insulin (Ins P) or leptin (Lep P). Following solubilization, cells lysates were immunoprecipitated with anti-hLBD antibodies and subjected to Western blotting. The blots were incubated with antibodies directed towards IRS-1, IRS-2 or p85, and the proteins were revealed by ECL®. IB, immunoblot; IP, immunoprecipitation.

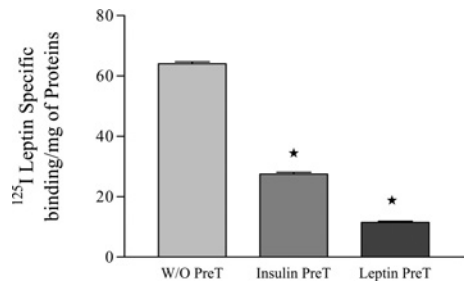

Figure 11 Effects of chronic leptin or insulin treatment on ObR number

Serum-deprived cells were pre-treated for 16 h without (W/O PreT) or with insulin (Insulin PreT) or leptin (Leptin PreT), and binding assays were performed on intact cells using ^{125}I-leptin. The results are expressed as leptin specific binding/mg of total cell proteins $\pm$ S.E.M. ($n = 4$); * indicates $P < 0.0001$.

a lesser extent (approx. 40 %), was observed following leptin pre-treatment (Figure 12).

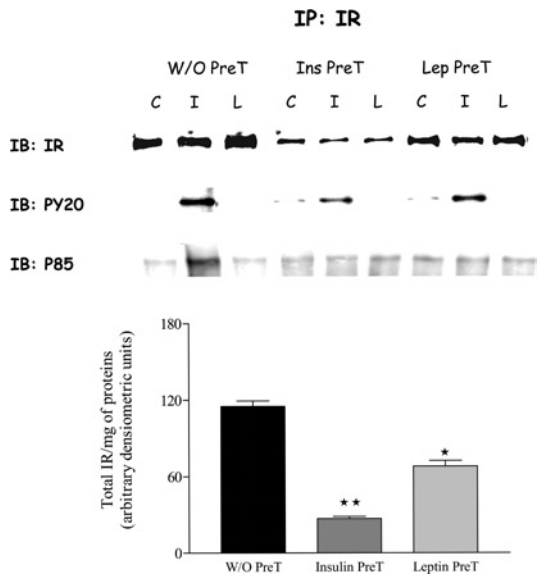

Figure 12 Effects of chronic leptin or insulin treatment on IR phosphorylation and number

Serum-deprived cells were incubated in the absence (C) or presence of insulin (I), or leptin (L) for 10 min following a 16 h pre-treatment without (W/O PreT) or with insulin (Ins PreT) or leptin (Lep PreT). Following solubilization, cells lysates were immunoprecipitated with anti-IR antibodies. The blots were incubated with antibodies directed towards IR, phosphotyrosine (PY20) or p85 subunit, and revealed by ECL®. For IR quantification, immunoblots with anti-IR antibodies were quantified using Scion Image and results were expressed as the total IR protein expression/mg of total cell proteins $\pm$ S.E.M. ($n = 4$). * and ** indicate $P < 0.001$ and $P < 0.0001$ respectively. IB, immunoblot; IP, immunoprecipitation.

DISCUSSION

Leptin and insulin receptors have in common a number of signalling pathways, such as IRS–PI3K and JAK2–STAT-3 [29,30,42]. The alteration of one of these signalling pathways, by inhibitors, such as inhibitors of PI3K [43,44], or by expressing ObRb lacking Tyr^{1138} which is crucial in the ObRb–STAT-3 interaction [21–23], leads to profound metabolic and energy balance control failures which may contribute to development of obesity and Type II diabetes. The interaction between leptin and insulin signalling pathways have been partially dissected *in vitro* in non-neuronal cells, such as hepatoma, L6 rat skeletal muscle and C2C12 cells [29,45,46]. In the present paper, we aimed to analyse the cross-talk between insulin and leptin in a human neuroblastoma-derived SH-SY5Y cell line.

SH-SY5Y cells underwent differentiation when stimulated by RA, which is accompanied by the over-expression of endogenous leptin receptor, as shown by immunocytochemistry, Western blotting and RT-PCR. These cells also express IR in an RA-independent manner. We focused our study on three major signalling cascades: IRS–PI3K, JAK2–STAT-3 and MAPK.

Both leptin and insulin have led to significant increase in STAT-3 tyrosine phosphorylation. Whereas the effect of leptin is well documented, the role of insulin on the tyrosine phosphorylation of STAT-3 has been controversial and dependent on the cellular model studied [30,47,48]. A slight tyrosine phosphorylation of STAT-3 in response to insulin in liver [48] has been reported, and another study has suggested that insulin phosphorylates STAT-3 on a serine residue, but not on tyrosine [47]. In the present study, we demonstrated a significant tyrosine phosphorylation of STAT-3 in response to insulin, most likely through JAK2, as previously suggested [30]. But we can not exclude a direct phosphorylation of STAT-3 by IR, as previously described for STAT-5B, where STAT-5B interacts with IR through its SH2 domain [49]. Furthermore, when cells were pre-treated with insulin, basal STAT-3 tyrosine phosphorylation was significantly increased and leptin (or insulin) acute stimulation did not further increase phosphorylation and became ineffective. Similar results were obtained after leptin pre-treatment. These results indicated that leptin or insulin pre-treatment did not lead to insulin or leptin-sensitizing effects respectively. A similar effect of insulin pre-treatment has been described in Huh7 cells, but these cells do not express endogenous leptin receptor and these results were obtained after over-expression of leptin receptor [42]. This contrasts with previous data obtained *in vivo* where leptin has an insulin-sensitizing effect, as shown by a rapid reduction in glucose and insulin levels in *ob*/*ob* mice after leptin treatment [35], or by an increase in insulin-dependent glucose utilization in normal rats treated with leptin [50]. Here, both pre-treatments, and specially insulin pre-treatment, increased basal STAT-3 tyrosine phosphorylation and the absence of insulin or leptin acute effect may be the consequence of the down-regulation of both leptin and insulin receptors and signalling in pre-treated SH-SY5Y cells. Previous results obtained in other human neuroblastoma cells, such as SK-N-MC or IMR32 cells, have showed that insulin pre-treatment increased ObRb expression at the level of mRNA [51]. It is worth noting that these

neuroblastoma cells were not differentiated, and the SH-SY5Y cells used in the present paper were differentiated by RA treatment and this may explain these differences. It is of note that previous studies that dealt with insulin and leptin cross-talk at the level of STAT-3 were performed on cells derived from peripheral tissues (such as hepatoma cells or hepatocytes) [29,48], and the present study, to our knowledge, is the first investigation describing such interaction in human neuroblastoma cells.

The other signalling pathway that may be an important site of leptin and insulin signalling cross-talk is the MAPK pathway. Insulin and leptin clearly increased phosphorylation of MAPK, but the combination of leptin and insulin significantly reduced MAPK phosphorylation as compared with the effect of insulin alone. The effect of leptin on insulin-induced MAPK phosphorylation may be due to the activation of a phosphatase, such as SHP-2 (SH2-domain-containing tyrosine phosphatase 2) [52,53] which is activated by leptin receptor through JAK2 and able to dephosphorylate IR and IRS-1/IRS-2. However, this hypothesis may be contradictory with the fact that SHP-2 is also able to activate insulin-dependent MAPK pathway through the docking protein GAB1 (Grb2-associated binder 1) [14]. After insulin pre-treatment, the basal MAPK phosphorylation was increased and the amplitude of leptin or insulin effect on MAPK phosphorylation was reduced. However, despite the increase in the basal MAPK phosphorylation both leptin and insulin maintained their effect. Therefore, only a weak desensitization was observed. Similar data were obtained with leptin pre-treatment. In both pre-treatments, the combination of insulin and leptin did not further alter MAPK phosphorylation in response to leptin or insulin which is probably associated with the fact that the pre-treatment has already activated phosphatase activity. The results obtained clearly show that the leptin or insulin pre-treatment did not totally abolished MAPK phosphorylation in contrast with STAT-3 phosphorylation, which was completely abolished in response to acute stimulation with insulin or leptin. One possible explanation is that the phosphorylation of MAPK requires less receptors to be activated.

The other major signalling cascade that may be considered important in insulin and leptin receptor cross-talk is the IRS–PI3K pathway. The role of insulin receptor in regulating PI3K has been largely studied *in vivo* and *in vitro* in numerous cellular models, whereas the role of leptin is still a matter of controversy and the mechanism of activation of PI 3 kinase following leptin stimulation is dependent upon cell type. In the present study in SH-SY5Y cells, we demonstrated that leptin is able to activate PI3K and preferentially through IRS-2, whereas insulin signals through IRS-1. The preferential activation of PI3K associated to IRS-2 by leptin has been also described in rat liver [54]. However, both leptin and insulin are able to activate PI3K through IRS-1 and IRS-2 respectively. This has been shown by immunoprecipitation study and by measuring PI3K activity associated to IRS-1 or IRS-2. Interestingly, we also show that p85 subunit of the PI3K co-immunoprecipitates with ObRb and this association is dependent upon leptin, but not insulin. This is the first evidence suggesting the presence of a multimeric complex, most likely involving ObRb, JAK2, IRS-2 and p85, following leptin stimulation. It is noteworthy that the combination of insulin to leptin and leptin to insulin significantly reduced the association of IRS-2 and IRS-1 with p85 respectively. This probably indicates a competition between IR–IRS-1 and ObRb–IRS-2 complexes towards p85 subunit, which also suggests that both receptors use the same cellular 'pool' of p85. Furthermore, the long-term pre-treatment with leptin or insulin increased basal PI3K activity and IRS-1 or IRS-2 association with p85. However, in these conditions acute stimulations with leptin or insulin tended to reduce PI3K activity and the association of IRS-1/IRS-2 with p85. This inhibitory effect of leptin and insulin may be attributed to the striking augmentation of phosphatase activities, as mirrored by the measurement of PTP-1B, SHP-1 and SHP-2 tyrosine phosphorylation, following chronic treatments (results not shown). In addition, leptin or insulin pre-treatment induced IRS-1 and IRS-2 down-regulation, which may contribute to the reduction of PI3K activation following acute leptin or insulin stimulation.

Finally, in addition to these complex interactions between the IR and ObRb downstream signalling pathways, we demonstrate that overexposure of cells to leptin down-regulates IR, and vice versa. This is the first study to support the fact that high concentrations of leptin are able to down-regulate IR and vice versa, in addition to the down-regulation of IRS-1 and IRS-2. These results may contribute to the understanding of the link between insulin resistance during hyperleptinaemia and the onset of metabolic pathologies, such as diabetes and obesity.

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